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Assessment of Alkaline Phosphatase on the Surface Membrane of Neutrophils by Immunofluorescence

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Expression of alkaline phosphatase (ALP) on the surface membrane of neutrophils (mNAP) was studied by immunofluorescence using an anti-ALP monoclonal antibody. Fluorescent intensity distribution of mNAP was analyzed using FACS (fluorescence-activated cell sorter). The mean fluorescent intensity (MFI) of the mNAP in this assay was well correlated with the neutrophil ALP (NAP) score demonstrated cytochemically (r = 0.832). mNAP levels in various hematological disorders were evaluated by % mNAP$^+$ cells and MFI. The levels in aplastic anemia and polycythemia vera were significantly higher, and in chronic myelocytic leukemia and paroxysmal nocturnal hemoglobinuria (PNH), the levels were significantly lower compared with the levels in healthy volunteers. Two-color immunofluorescence with anti-ALP and anti-CD16 showed that the PNH clone was essentially negative for mNAP, whereas residual normal neutrophils (CD16$^+$) had levels slightly higher than those in normal individuals. Highly reproducible results were obtained in the blood samples which were stored at 4°C for at least 24 hr without any treatment prior to immunofluorescent staining. No degradation of fluorescent intensity was seen 4 days after staining and fixation. The mNAP assay is simple, without subjective evaluation for quantification, and is useful for differential diagnosis of hematological disorders. Am. J. Hematol. 60:12–18, 1999.

Key words: neutrophil; alkaline phosphatase; monoclonal antibody; laboratory methods; flow cytometry

INTRODUCTION

Alkaline phosphatase (ALP: E.C.3.1.3.1) is an enzyme that catalyzes the hydrolysis of various monophosphate esters. Most of the enzyme (~80%) is stored on the luminal side of the secretory vesicles in cytoplasm, and the remaining is expressed on the surface membrane in unstimulated neutrophils [1–3]. ALP is a member of the growing family of membrane-associated proteins anchored to the outer leaflet of the lipid bilayer via a glycosyl-phosphatidylinositol (GPI) moiety [4–6]. At least four different isozymes are coded by distinct ALP genes in humans [7–10], and neutrophil ALP belongs to liver/bone/kidney-type ALP [11].

Although the biological role of this enzyme in neutrophils has remained unclear, usefulness of the examination of neutrophil ALP (NAP) activity for differential diagnosis of certain hematological disorders has been well documented [12]. Abnormally low NAP scores are preferentially seen in chronic myelogenous leukemia (CML) and paroxysmal nocturnal hemoglobinuria (PNH). Conversely, high NAP scores are frequently found in inflammatory leukocytosis, polycythemia vera (PV) and aplastic anemia (AA), and occasionally in idiopathic myelofibrosis (IMF). NAP activity is generally demonstrated using the cytochemical method [13], but delicate cautions are needed in the staining procedure [14], and assessment of the enzyme content depends to a certain degree on subjective evaluation by the observer.

Flow cytometry has increasingly been used for study on expression of various surface molecules on blood

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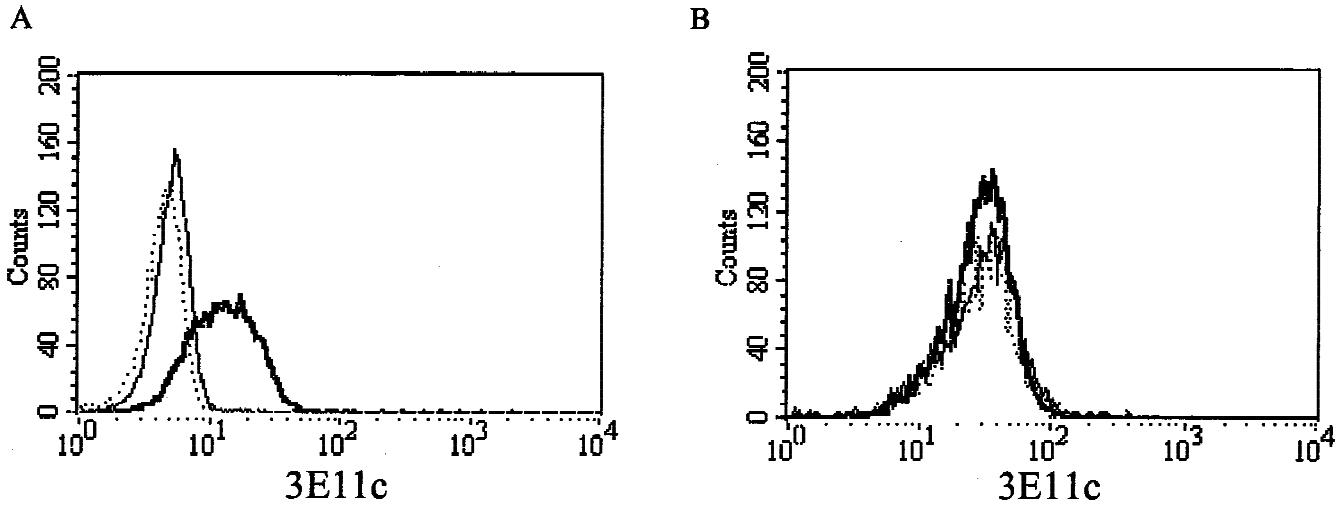

Fig. 1 (A) Detection of mNAP on neutrophils employing indirect immunofluorescence using biotinylated 3E11c (b-3E11c). The reactivity was completely inhibited by adding 6-fold amount of purified 3E11c. Dotted, bold, or solid lines indicate negative control, b-3E11c, or b-3E11c + purified-3E11c, respectively. (B) Effect of storage of blood samples on mNAP. Reactivity of mNAP in the samples stored at 4°C for 0, 12, and 24 hr as indicated by solid, bold, and dotted lines, respectively, is shown.

cells. In this study, expression of ALP on the surface membrane of neutrophils (mNAP) was investigated in healthy donors and patients with various hematological disorders by immunofluorescence using an anti-ALP monoclonal antibody. We compared the expression of mNAP with NAP activity demonstrated through cytochemical methods, and evaluated the diagnostic value of the mNAP assay.

MATERIALS AND METHODS

Anti-ALP monoclonal antibody. Anti-ALP monoclonal antibodies were produced by immunization with purified bone ALP [15]. In this study, the antibody 3E11c, strongly reacting with leukocyte ALP as well as bone ALP, was used. The immunoglobulin (Ig)G fraction was purified from supernatant in a culture of hybridoma cells using the salting-out method and affinity chromatography with protein A.

For biotinylation, a solution containing 3E11c was dialyzed three times in sodium bicarbonate buffer and mixed with N-hydroxysuccinimide (NHS)-biotin (Pierce Chemical Co., Rockford, IL) to a final concentration of 60 μg/ml for 4 hr at room temperature, and was again dialyzed three times in 10 mM phosphate-buffered saline (PBS) containing 0.1% sodium azide.

Blood samples. Peripheral blood samples were collected after informed consent from 36 normal volunteers and 86 patients with hematological disorders: Ten cases of CML; 12 cases of PV; 7 cases of essential thrombocythemia (ET); 5 cases of IMF; 21 cases of PNH; 16 cases of AA; and 15 cases of myelodysplastic syndrome (MDS).

Immunofluorescence procedures. Ten microliters of the appropriately diluted biotinylated 3E11c (b-3E11c) were added to 100 μl of unfractionated blood samples (final 10 μg/ml), followed by indirect immunofluorescence procedures using phycoerythrin (PE)-conjugated streptavidin as the second antibody. Next, the samples were fixed, and erythrocytes were lyzed with the fluorescence-activated cell sorter (FACS) lysing solution (Becton-Dickinson Co., Mountain View, CA). Following this, the cells were washed with PBS containing 0.2% sodium azide, and were resuspended in the same buffer. The forward and the side scatter gate was defined on neutrophils with FACScan (Becton-Dickinson Co.), and the expression of mNAP was analyzed.

Two-color immunofluorescence with 3E11c and anti-CD16 was performed for examination of mNAP expression on each of normal ($CD16^+$) and abnormal ($CD16^-$) neutrophils in samples from PNH cases. At first, the cells were incubated with b-3E11c, and then stained with a mixture of streptavidin-PE and CD16-fluorescein isothiocyanate (FITC).

Cytochemical detection of NAP. NAP activity was demonstrated according to the method described by Tomonaga [14,16], one of the standard azo dye methods using naphthol AS-MX phosphate as a substrate. Blood films were prepared immediately or within 3 hr after collection of heparinized venous blood and dried in air for 15–30 min. Then, they were fixed with fixative for 5 sec, and washed with running water for 15–30 sec. After drying in air, the blood films were incubated at 37°C for 2 hr in the freshly prepared substrate solution, counterstained with safranin O and observed under a light mi-

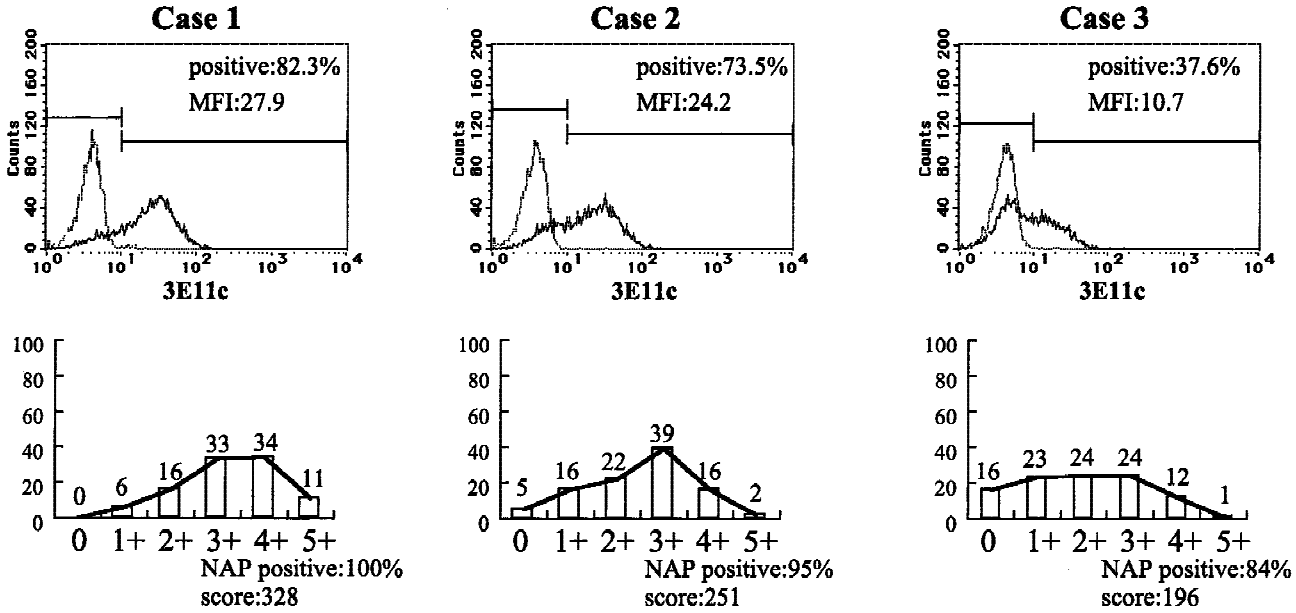

Fig. 2. Comparison of mNAP expression in normal volunteers with NAP activity demonstrated by the azo dye method (Tomonaga). Staining intensity distribution of mNAP and NAP are shown on the histograms.

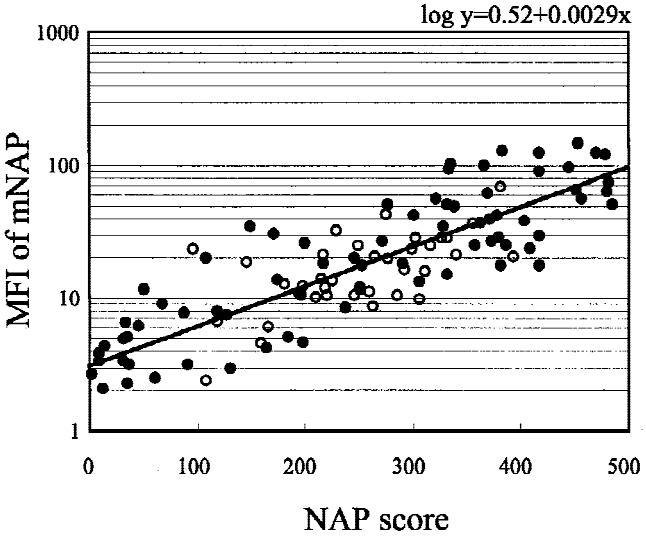

Fig. 3. Correlations between mNAP and NAP in 107 samples including 36 normal volunteers (open circles) and 71 patients with hematological disorders (closed circles). Pearson's sample correlation coefficient (r) is 0.832 ($P < 0.001$).

croscope. One hundred segmented and stab forms of neutrophils were rated from 0 to 5+ on the basis of the intensity of the precipitating dye. The number of cells with the precipitating dye and the sum of ratings in 100 neutrophils were recorded as NAP rate and NAP score, respectively.

Statistics. Pearson's correlation coefficient with Fisher's Z-transformation was used for the correlation between mNAP and NAP.

RESULTS

Reactivities of Anti-ALP Monoclonal Antibody, 3E11c

Reactivity of 3E11c with normal neutrophils was examined by indirect immunofluorescence. As shown in Figure 1A, neutrophils showed variable fluorescent intensities and formed a relatively broad-based peak on the histogram of mNAP. The reactivity of b-3E11c was completely inhibited by adding 6-fold amounts of purified 3E11c.

We also examined mNAP expressions in the blood samples from two healthy volunteers, which were stored at 4°C for 0, 12, 24, and 48 hr after collections, respectively. As shown in Figure 1B, no significant changes in mNAP expression were seen in any of the samples from each volunteer, but the leukocyte counts in those stored for 48 hr were diminished due to aggregation. Moreover, after staining and fixation, no degradation was recognized in fluorescent intensity of mNAP for at least 4 days in the samples stored at 4°C (data not shown).

For assessment of mNAP expression, we determined two indices, % $mNAP^+$ cells and mean fluorescent intensity (MFI). The 90% confidence interval for the $mNAP^+$ cells in samples from 36 normal volunteers was 17.3 to 88.7%, and those for MFI was 5.7 to 40.9, which were used as the normal ranges.

Correlations Between mNAP and NAP

Expression of mNAP was compared with NAP activity demonstrated by the azo dye methods in three normal volunteers. As shown in Figure 2, the fluorescent inten-

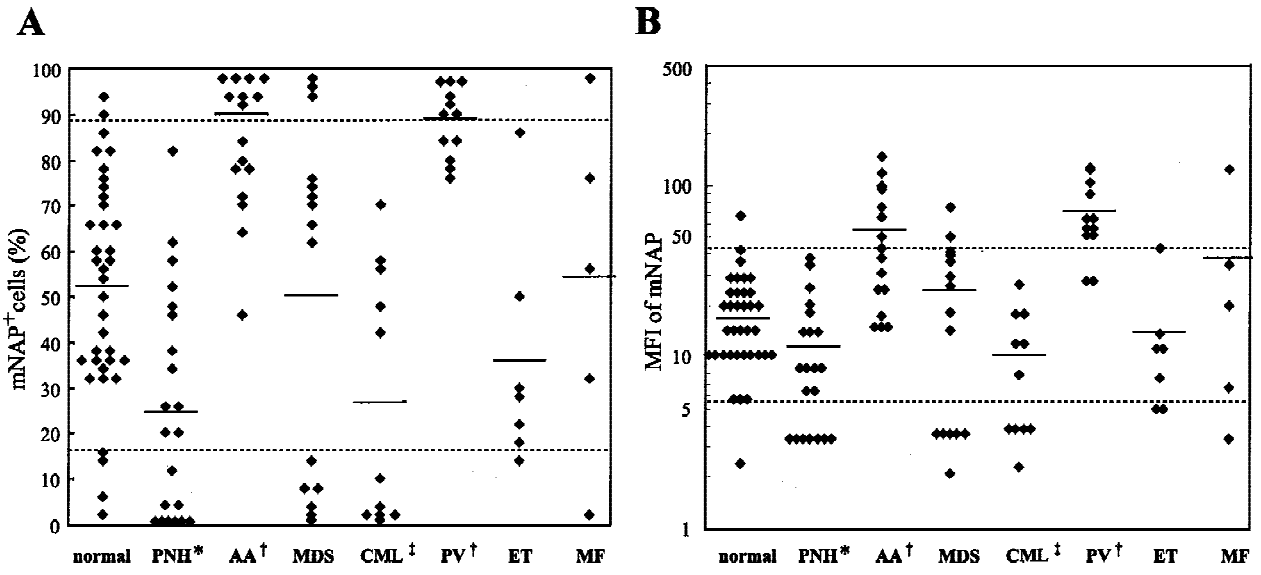

Fig. 4. Percentages of mNAP$^+$ cells (A) and MFIs of mNAP (B) in hematological disorders. Ninety percent confidence intervals in healthy individuals are shown by dotted lines (17.3% to 88.7% for percent mNAP$^+$ cells and 5.7 to 40.9 for MFI), respectively. *, ‡: Significantly lower ($P < 0.01$ and $P < 0.02$, respectively), †: Significantly higher ($P < 0.0005$) than the normal level.

sity distribution curve of mNAP was quite similar to the pattern of the histogram for NAP ratings determined by Tomonaga's method [14]. On the other hand, such approximate similarity in the distribution pattern was not obtained between mNAP and NAP activity demonstrated by Kaplow's method [12–14], because in the latter method, a higher proportion of neutrophils in most cases of normal volunteers were rated as zero, whereas fewer were rated as three or four. Therefore, in the following studies, expression of mNAP was compared with the NAP activity demonstrated using Tomonaga's method.

The relationship between mNAP expression and NAP activity was investigated in 107 samples from normal volunteers and patients with hematological disorders. MFI in mNAP was well correlated with the NAP score ($r = 0.832$) as shown in Figure 3. The percent of mNAP$^+$ cells was also correlated with % NAP$^+$ cells ($r = 0.807$).

Expression of mNAP in Hematological Disorders

Figure 4 shows mNAP levels, % mNAP$^+$ and MFI, in 86 patients with hematological disorders. mNAP levels in samples from patients with AA and PV were significantly higher than those in normal volunteers. In contrast, patients with PNH and CML demonstrated noticeably lower levels compared with normal volunteers. In four CML cases whose mNAP levels were dotted within the normal range, the leukocyte counts had been controlled to less than 9×10^9/l through treatment with hydroxyurea and/or interferon-α, with the exception of one case in the accelerated phase. Although approximately half of the PNH cases had mNAP levels (% mNAP$^+$ and MFI) within the normal range, the fluorescent distribution curves formed two distinct peaks (Fig. 5A), which appeared to correspond to mNAP$^-$ cells (PNH clone) and mNAP$^+$ cells in these patients, and were different from those with a broad-based peak seen in normal volunteers. (Fig.2). To identify the PNH clone and evaluate mNAP levels in the PNH clone and in the residual normal neutrophils, two-color immunofluorescence was performed using b-3E11c and anti-CD16 reacting with another GPI-anchored protein on neutrophils. As shown in Figure 5B, the PNH clone and apparently normal neutrophils were clearly separated by the two-color immunofluorescence in all PNH cases except for those without a recognizable mNAP$^+$/CD16$^+$ population. On the other hand, there were no mNAP$^-$/CD16$^-$ cells in normal volunteers. As was expected, mNAP in the PNH clone from all cases showed essentially negative reaction for mNAP. On the other hand, both % mNAP$^+$ cells and MFI of mNAP in CD16$^+$ neutrophils from patients with PNH showed slightly higher levels compared with neutrophils in normal volunteers (Fig. 5C,D). The mNAP levels were also low in CML, however, the mNAP histogram showed a monomodal pattern without a deficient expression of CD16 in CML neutrophils, differing from PNH neutrophils.

Some patients with MDS showed abnormally low or high mNAP levels, suggesting heterogeneity of the disease, although further studies including association of the mNAP levels with subtypes of MDS are needed.

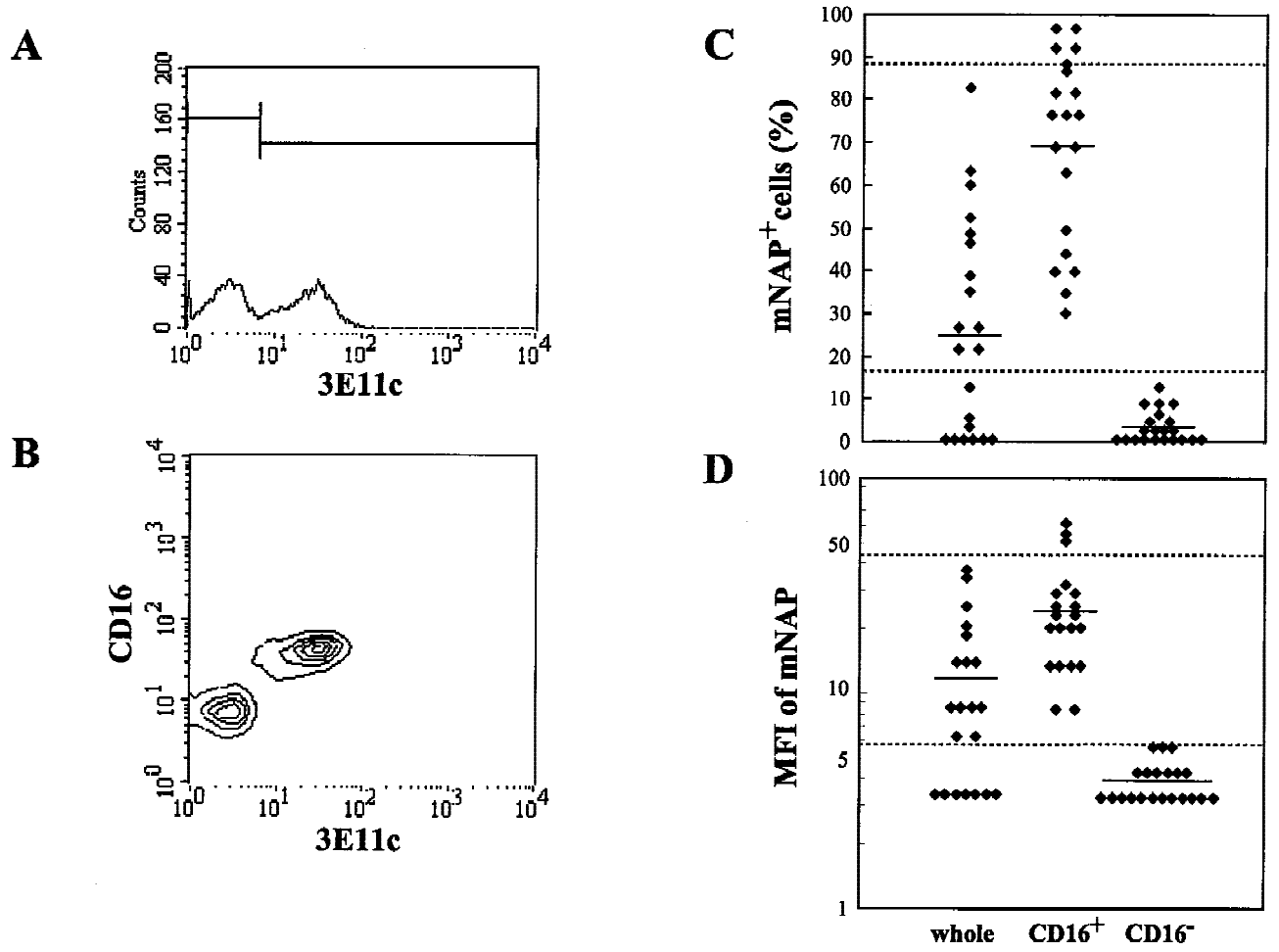

Fig. 5. Expression of mNAP in PNH. (A) Neutrophils from a patient with PNH showed a bimodal pattern on the mNAP histograms. (B) Two-color immunofluorescence with anti-ALP and anti-CD16 in the same patient in (A). The PNH clone and apparently normal neutrophils are clearly separated from each other. (C,D) Expression of mNAP on the whole neutrophils, and $CD16^+$ and $CD16^-$ neutrophils in PNH. mNAP levels in $CD16^+$ neutrophils was slightly elevated compared with those in neutrophils from normal volunteers ($P < 0.02$). Horizontal bars indicate the mean level of % $mNAP^+$ cells and MFI.

DISCUSSION

NAP activity has been demonstrated using cytochemical methods, and the usefulness of the examination in the differential diagnosis of certain hematological disorders is well documented [12,13]. The cytochemical azo dye method has been improved and standardized, but delicate cautions in fixation and staining of the blood films including time, temperature, and pH are still needed for obtaining satisfactory results [14]. Moreover, quantification of NAP activity depends to a certain degree on the subjective evaluation of observers.

In this study, we assessed the expression of ALP on the surface membrane of neutrophils, mNAP, and compared mNAP expression with NAP activity demonstrated by the azo dye method. The % $mNAP^+$ and MFI in normal volunteers and those with hematological disorders were correlated with NAP scores and the percentages of NAP^+ cells (NAP rates), respectively ($r = 0.832$ and 0.807). Moreover, the fluorescent intensity distribution curve of mNAP closely resembled the pattern in the histogram of NAP rating determined by Tomonaga's method.

We also examined reproducibility in the mNAP assay, and effect of storage of the samples on the assay. The assay using standard indirect immunofluorescence can be performed with little technical difficulty, and highly reproducible results were obtained when the same FACScan was used. In addition, no significant change was recognized in mNAP expression in the blood samples that were stored at 4°C for at least 24 hr without any treatment after blood collection. After staining and fixation, the sample can be stored at 4°C for at least 4 days without significant change in fluorescent intensity. These features of the assay appear to be particularly optimal for examining many samples from various institutes in a clinical laboratory center.

The percent of mNAP$^+$ and the MFI in patients with AA and PV or CML and PNH were significantly higher or lower than those of normal volunteers, respectively, as described for NAP. Our findings are consistent with those described by Rambaldi et al. [17] who also examined expression of ALP on neutrophils in hematological disorders using flow cytometry. These findings indicate that the mNAP assay is useful for differential diagnosis of these disorders. Although a certain degree of overlap was seen between these patients and normal volunteers, the mNAP levels at presentation were low in all of our patients with CML. Samples from approximately half of the patients with PNH also showed mNAP levels within the normal range, but the fluorescent distribution curves in a single-color immunofluorescence test showed two distinct peaks corresponding to the PNH clone and apparently normal neutrophils in these patients. The separation of these two populations was even clearer on a two-color immunofluorescence test using 3E11c and anti-CD16.

ALP is mainly (~80%) stored on the luminal side of the secretory vesicles, the highly mobilizable storage organelles in cytoplasm and the remaining (~20%) are expressed on the surface membrane in unstimulated neutrophils. When neutrophils are stimulated, these vesicles are mobilized and fuse with plasma membrane, followed by the translocation of ALP to the outside of plasma membrane [1–3]. Both intracytoplasmic and surface ALP are anchored to membranes via GPI moiety, and their expression is lost or reduced due to deficiency of the GPI anchor in PNH [4,18,10]. It has been described that neutrophil stimulators such as N-formyl-Met-Leu-Phe [1,20] or granulocyte-colony stimulating factor (G-CSF) [21] up-regulate transcription of the ALP gene and facilitate movement of ALP to the surface membrane. Since serum G-CSF levels are elevated in many patients with AA [22,23], strong expression of mNAP in AA appears to be associated with the mechanism described above. Increased or decreased transcription of the ALP gene in neutrophils has been described for PV or CML, respectively [24]. Our results for mNAP expression in these diseases are consistent with the levels of ALP transcription. Results of mNAP assay strongly correlated with those of NAP, suggesting that surface expressions of ALP in most cases are parallel to the intracytoplasmic expression; however, some dissociation was observed in a small portion of the cases studied. In these cases, the dissociation may be due to a difference in the distribution of ALP between intracytoplasm and surface membrane, although further research is needed to study the dynamics of ALP in neutrophils.

In this study, we demonstrated that mNAP expression detected by flow cytometry is well correlated with NAP activity determined using the cytochemical method. The mNAP assay can be performed with little technical difficulty and high reproducibility. The assay is therefore useful for clinical practice, and will also be beneficial in the studies of the dynamics of ALP in neutrophils and association of ALP with neutrophil functions.

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